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Characterization of influenza virus variants with different sizes of the non-structural (NS) genes and their potential as a live influenza vaccine in poultry

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ABSTRACT

From a stock of A/turkey/Oregon/71-delNS1 (H7N3) virus, which has a 10 nucleotide deletion in the coding region of the NS1 gene, we found that several variants with different sizes of NS genes could be produced by passaging the virus in 10- and 14-day-old embryonating chicken eggs (ECE), but not in 7-day-old ECE or Vero cells. We were able to rescue the reassortant virus that has different sizes of the NS genes and confirmed that those NS genes are genetically stable. By conducting *in vivo* studies in 2-week-old chickens, we found two plaque purified variants (D-del pc3 and pc4) which can be used as a potential live-attenuated vaccine. The variants were highly attenuated in chickens and did not transmit the virus from infected chickens to uninoculated cage mates. At the same time, the variants induced relatively high antibody titers which conferred good protection against a high dose heterologous virus challenge. Our study indicates that naturally selected NS1 deletion variants might be useful in the development of live-attenuated influenza vaccines in poultry. Furthermore, deletion in the NS1 protein can be potentially useful as a negative marker for a differentiating infected from vaccinated animals (DIVA) approach.
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1. Introduction

Influenza A virus has 8 RNA segments encoding at least 10 proteins [1]. Among these proteins, most are structural proteins and are found in the virions. Although recently identified PB1-F2 is a non-structural protein expressed by many influenza viruses [2], the non-structural protein 1 (NS1) protein is the only known non-structural protein expressed by all influenza viruses [3]. The non-structural (NS) gene segment is the smallest in the influenza genome, typically 890 nucleotides (nt) in size and encodes for two different proteins, NS1 and nuclear export protein (NEP). The NEP coding sequence partially overlaps the NS1 protein at the amino terminal end and the carboxy terminal end is a result of alternative splicing of its mRNA. The NEP protein, previously known as NS2, was thought to be a non-structural protein, but it is present in small amounts in the virions in association with the ribonucleoprotein (RNP) through interaction with the M1 protein [1]. In most viruses, the NS1 protein consists of 230 amino acids (aa) and the NEP protein of 121 aa. The NS1 protein can be divided into two major domains which are the N-terminal RNA binding domain and the remaining effector domain [4]. This protein is a multifunctional protein with regulatory effect on a variety of host cell functions including suppression of innate immunity by preventing host cell mRNA processing [5], blocking nuclear export of polyadenylated cellular transcripts [6,7], and inhibiting type I interferon (IFN) induction and/or production [8]. In addition to its inhibitory role in innate immunity, it was recently also shown that NS1 protein can inhibit adaptive immunity by attenuating human dendritic cell (DC) maturation and the capacity of DCs to induce T-cell responses [9].

Live-attenuated virus vaccines have several distinct advantages over inactivated vaccines such as triggering mucosal immune responses and inducing a cell-mediated immunity, which may

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give vaccinees a more cross-protective and longer-lasting immunity [10,11]. Furthermore, live-attenuated influenza virus vaccines can potentially be administered by aerosol or water exposure, like Newcastle disease virus vaccines, which provides for cost effective mass administration of the vaccine, and is a major advantage over current whole virus adjuvanted killed vaccines which must be administered parenterally by needle injection. Previous studies have shown that the blocking or reducing of the function of the NS1 protein can effectively attenuate the virus, in large part because it allows the host to generate a strong interferon response to which influenza viruses are intrinsically sensitive. Thus, viruses producing defective NS1 proteins show highly attenuated phenotypes both *in vitro* and *in vivo*, and might be useful as live-attenuated vaccine candidate strains [12–14]. It was also shown that viruses lacking the NS1 gene are potent stimulators of human DCs and therefore can be potent immunogens [9]. In addition, since the NS1 protein is not incorporated into virions, alterations of this protein would not change the antigenicity of the influenza virion itself. Thus, the influenza virus NS1 protein is an excellent target for attenuation by genetic manipulation.

A/turkey/Oregon/71 (TK/OR/71) (H7N3) virus, which is a low virulence strain isolated from turkeys in 1971, exists as two genetically distinct stocks [15]. One stock, named TK/OR/71-SEPRL, is a low-passage stock of the field isolate that encodes a full-length NS1 protein of 230 aa. The other stock, referred to a TK/OR/71-delNS1, has unknown passage history and has a 10 nt deletion in the middle of the NS gene which results in producing a truncated NS1 protein of 124 aa [16]. The NEP protein open reading frames (ORFs) are intact in both strains. TK/OR/71-delNS1 is a strong inducer of IFN with 20-fold difference compared to TK/OR/71-SEPRL [17]. Clear differences in pathogenicity of these two viruses were observed in 1-day-old and 4-week-old chicken infection studies [18]. In the aforementioned study, TK/OR/71-delNS1 was highly attenuated compared to TK/OR/71-SEPRL and did not transmit efficiently from infected chickens to uninoculated cage mates, which showed the possibility of using the natural NS1 deletion variants, TK/OR/71-delNS1, as a potential live vaccine. However, we found that the NS gene of TK/OR/71-delNS1 was not genetically stable and variants with NS gene of different sizes and with different biological characteristics could be generated directly from this virus. This study describes the identification and characterization of these NS-deletion variants and the evaluation of individual NS variants as live influenza virus vaccine candidates.

2. Materials and methods

2.1. Viruses

The viruses used in this study were obtained from the repository of Southeast Poultry Research Laboratory (SEPRL), Athens, GA. The passage history of the TK/OR/71-delNS1 stock is unknown and we used the original Vero cell culture supernatant stock (originally kindly provided by Peter Palese, Mount Sinai School of Medicine, New York) for the study. TK/OR/71-SEPRL and A/chicken/NJ/150383-7/02 (H7N2) used in the challenge studies were passaged once in 10-day-old specific pathogen-free embryonating chicken eggs (ECE) to make working stocks of the virus.

2.2. TK/OR/71-delNS1 virus passage in Vero cells and 7-, 10-, and 14-day-old embryonating eggs

The TK/OR/71-delNS1 stock was passaged five times in Vero cells and ECE of different ages, respectively. Vero cells were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum. The cells were seeded (approximately 10^6 cells/well) in six-well tissue culture plates the day before infection. The cells were infected with 1:1000 dilution of the virus from each passage and infectious supernatants were collected 2 days after infection. Virus propagation in the allantoic cavity of SPF ECEs was done as previously described [19].

2.3. RNA extraction and NS-gene specific RT-PCR, cloning and sequencing

Viral RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA) from infectious cell supernatants or allantoic fluids from ECE as previously described [20]. Standard RT-PCR was carried out with the Qiagen one-step RT-PCR kit (Qiagen) with NS gene-specific primers NS + 1: 5′-AGCAAAAGCAGGGTGACAA-3′ and NS-890: 5′-AGTAGAAACAAGGGTGTT-3′. The PCR products were separated on an agarose gel by electrophoresis and amplicons were subsequently excised from the gel and extracted with Qiagen gel extraction kit (Qiagen). The purified PCR products were cloned into the TOPO-TA vector following the manufacturer's protocol (Invitrogen). Sequencing was performed with a 3730 automated sequencer (PerkinElmer, Waltham, MA) or ABI PRISM 377 DNA sequencer at the SEPRL sequencing facility or Molecular and Cellular Imaging Center at The Ohio State University.

2.4. Rescue of recombinant influenza viruses with NS genes of different sizes

Wild-type and mutant NS genes were cloned into pHH21 vector between the promoter and terminator sequences of RNA polymerase I [21,22]. Plasmids expressing the remaining seven influenza virus genes from influenza A/WSN/33 virus were kindly provided by Yoshihiro Kawaoka, University of Wisconsin, Wisconsin. Other plasmids used in recombinant influenza virus rescue were described in our previous studies [23,24]. Recombinant viruses were generated by DNA transfection as previously described with minor modification [22,25]. Briefly, 293T cells were transfected with 1 μg of each of the eight viral RNA genes expressing plasmids and four expression plasmids for the influenza virus proteins NP PA, PB1, and PB2 with the use of Lipofectamine 2000 reagent (Invitrogen). Forty-eight hours after the transfection, the supernatants were collected and subsequently inoculated into 10-day-old ECE for virus propagation. At 72 h post-inoculation, allantoic fluids containing recombinant viruses were harvested and stored at −70 °C for additional experiments. The identity of the NS gene was confirmed by sequencing.

2.5. Cloning of the influenza virus variants by plaque assay

To biologically purify influenza virus variants that have different NS genes from a mixed virus population, we conducted screening of progeny viruses that arose during passage in 10- and 14-day-old ECE by plaque purification in chicken embryo fibroblast (CEF) cells followed by RT-PCR and sequencing. RNA extraction, RT-PCR, and direct sequencing were done as described above.

2.6. Western blot analysis

Wells (in six-well plates) of confluent Vero, MDCK, and CEF cells were mock infected or infected at a multiplicity of infection (MOI) of 2 with wild-type and NS variant viruses. At 8 and 12 h post-infection (p.i.), cells were lysed in 100 μl of radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and 10% of the lysates were loaded on a 15% sodium dodecyl sulfate

(SDS) polyacrylamide gel. Separated proteins were transferred to a membrane and subjected to Western blot analysis with rabbit polyclonal anti-NS1 and anti-NP antibodies [14]. Goat anti-rabbit immunoglobulin G (IgG) (H + L) peroxidase antibody was used as a secondary antibody (Boehringer Mannheim, Germany). Western blots were developed with a chemiluminescent reagent (Dupont-NEN, Wilmington, DE).

2.7. Virus growth analysis in Vero and CEF cells

Vero cells were maintained as described above. Primary CEF cells were prepared from 10-day-old eggs [26]. The cells were seeded (approximately 2×10^6 cells/well) in six-well tissue culture plates the day before infection. The cells were then infected at 0.01 MOI. After 1-h incubation, the inoculum was removed and replaced with media with the addition of trypsin (0.05 and 0.35 ug/ml for Vero and CEF cells, respectively). Cells were observed daily for cytopathic effects (CPE). Aliquots of supernatants were removed at 24, 48, and 72 h p.i. for titration. RNA was extracted from 200 μl of supernatants using the RNeasy Mini kit (Qiagen). One-tube real-time RT-PCR was performed using the Qiagen one-step RT-PCR kit (Qiagen) in a 25-μl reaction mixture containing probe-specific for the matrix gene [20]. For quantitation, samples were run together with known amounts of control viral RNA. Standard curves were generated with those control viral RNAs and the amount of RNA in the samples was converted into EID_{50}/ml by interpolation [20,21].

2.8. In vivo pathogenesis and challenge-protection studies

The virulence of four plaque-purified variants was determined in 2-week-old SPF chickens (Charles River Laboratories Inc., Wilmington, MA). The pathogenicity of TK/OR/71-SEPRL and TK/OR/71-delNS1 was previously described in detail [18]. In these experiments, 10 birds were infected with 10^6 EID_{50}/0.2 ml of virus by intranasal inoculation. Four contact-control birds were introduced to the same cage with infected birds 1 day after infection. Four infected birds were euthanized on 3 days p.i. to collect tissues for histopathology and immunohistochemistry (IHC). Collected tissues were fixed by submersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were made at 5 μm and were stained with hematoxylin and eosin (HE). A duplicate 4-μm section was immunohistochemically stained by first microwaving the sections in Antigen Retrieval Citra Solution (Biogenex, San Ramon, CA) for antigen exposure. A monoclonal antibody (P13C11) specific for a type A influenza virus nucleoprotein, developed at SEPRL, was used as the primary antibody for a streptavidin–biotin–alkaline phosphatase complex-based IHC method as previously described [27].

Tracheal and cloacal swabs were collected from all the birds on 2 and 4 days p.i. (1 and 3 days post-contact for sentinel control birds). Individual swabs were placed in 1.5 ml of sterile phosphate-buffered saline (PBS) containing gentamycin (1 mg per 100 ml). RNA was extracted from the tracheal and cloacal swabs with RNeasy Mini kit (Qiagen). The virus was quantitated by real-time RT-PCR as described above. Birds were observed for 2 weeks for clinical signs and sera were collected at 2-week p.i. to check specific influenza virus antibody responses by the hemagglutinination inhibition (HI) test [19].

Two weeks after infection (at 4 weeks of age), the remaining six infected (or live vaccinated) and additional six uninfected (unvaccinated) control birds were challenged with 10^6 EID_{50}/0.2 ml of heterologous H7N2 virus (A/CK/NJ/150383-7/02) by intranasal inoculation. We collected tracheal swabs at 3 and 5 days post-challenge from both vaccinated and unvaccinated challenged-control birds. Swab collection and titration were done as described above. Birds were observed for 10 days for clinical signs and sera were collected at the end of the experiment to check antibody responses by the HI test.

3. Results

3.1. Identification of NS genes of different sizes after passaging the TK/OR/71-delNS1 virus in 10- and 14-day-old eggs

We conducted NS gene specific RT-PCR with RNA extracted from delNS1 and SEPRL stocks of TK/OR/71 virus. In gel electrophoresis of the RT-PCR product, we observed a faint band which was smaller (approximately 700 bp) than the expected size (880 bp) of the NS gene from the delNS1 stock and not from the SEPRL stock (Fig. 1a). Based on previous studies that demonstrated increased IFN inducibility of both cultured chicken embryo cells and of intact ECE with increased age [28], the delNS1 virus was passaged in ECE of different ages as well as in Vero cells which are constitutively defective in producing IFN [29]. NS gene-specific RT-PCR was done with RNA extracted from allantoic fluids or supernatants of each passage (Fig. 1b). We observed no or a slight increase in intensity of smaller size bands when we passaged the delNS1 virus in Vero cells (data not shown) and 7-day-old ECE, respectively. In contrast, when we passaged the delNS1 virus in 10- and 14-day-old ECEs, the appearance of different size smaller bands was more obvious and 14-day-old eggs generated smaller size bands in fewer passages than the 7- or 10-day-old eggs.

By cloning and subsequent sequencing, we were able to identify more than 20 different NS genes of different sizes and the schematic diagram of those NS genes is shown in Fig. 2a. These NS genes were named as double-deletion (D-del) genes because they were derived

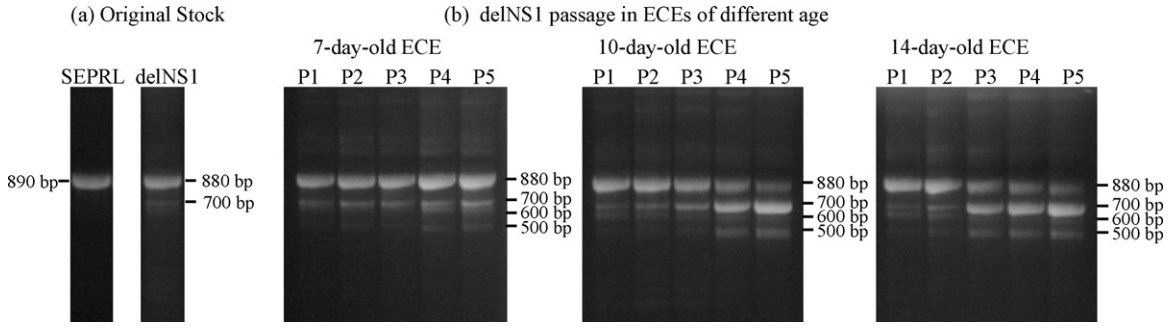

Fig. 1. Identification of NS genes of different sizes after passaging the TK/OR/71-delNS1 virus in embryonating chicken eggs (ECEs) of different ages. NS gene specific RT-PCR was conducted with RNAs extracted from original stock (a) and passaged (b) viruses and the amplification products were separated on a 1.5% agarose gel by electrophoresis. Number of passages are indicated on the top.

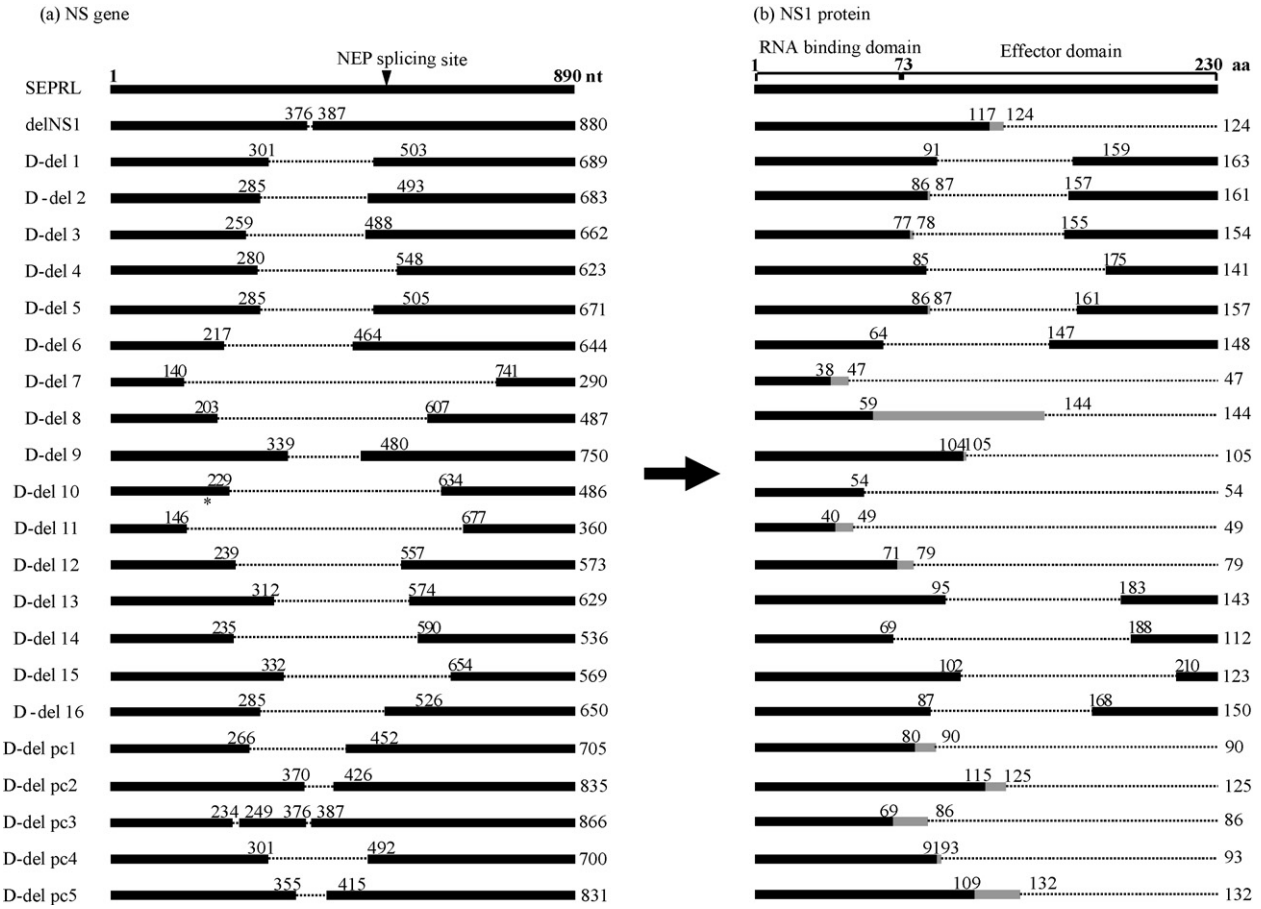

Fig. 2. Schematic diagram of the NS genes (a) and predicted NS1 protein (b) that were identified by cloning and subsequent sequencing. Black (thick) line: same nt or aa sequence compared to wild-type virus; dash line: deleted nt or aa sequence; arrow (in (a)): NEP splicing site; asterisk (in D-del 10 gene): premature stop codon. The carboxyl end of some of the NS1 proteins contains amino acid residues different from wild type (in gray color) due to a frame shift in the open reading frame.

from the stock that already had a 10 nt deletion. Most of the D-del NS genes sequenced have a large deletion in the middle of the NS gene. Among different NS genes, nine D-del NS genes are missing the NEP 3′ splicing site due to a large deletion in the middle of the NS gene. D-del pc3 NS gene has two separate regions of deletion because, in addition to one deleted region which is exactly the same as that of delNS1, it has another 14 nucleotide deletion upstream of the original parental deletion. A schematic diagram of predicted NS1 proteins based on NS gene sequence is shown in Fig. 2b. All the NS1 proteins retain an intact or partial double-stranded RNA binding domain, and some of them lost their entire effector domain while others retained a portion of the effector domain.

3.2. Rescue of reassortant viruses that have different size NS genes

We introduced the different size NS genes one by one in the backbone of a control influenza virus using 12 plasmid-based reverse genetics. We were able to rescue recombinant infectious viruses that have all the different sizes of the NS genes except D-del 5 and 16 genes and all D-del NS genes (D-del 4, 7, 8, 10–15) that lost the splicing site and part of the coding region of the NEP gene. Once the viruses were rescued, we passaged those recombinants in 10-day-old eggs to test if their NS genes are genetically stable. Mutant NS genes were genetically stable with the exception of the recombinant virus that had its NS gene derived from the original delNS1 virus. With this virus, we observed a pattern similar to that seen when we passaged the parental delNS1 virus in which the emergence of different size of deleted NS genes was obvious after a few passages (data not shown). Thus, it appears that most D-del NS genes are competent at least in the *in vitro* system we used as far as the NEP ORF is intact and they were directly derived from an unstable NS gene of original delNS1 virus.

3.3. Biological purification and characterization of the NS1 deletion variants

We were able to plaque purify the five variants (D-del pc1–pc5) and the schematic diagram of their respective NS genes and NS1 proteins is shown in Fig. 2. We passaged these *in ovo* selected variants at least five times in 10-day-old eggs and confirmed that those NS genes are stable and do not produce additional variants. By conducting Western blot analysis, we confirmed the expression of the truncated NS1 protein by the variants. We were able to detect more NS1 protein expression from infected Vero cells (Fig. 3) than from MDCK or CEF cells (data not shown). Even in Vero cells, we were not able to detect NS1 protein of D-del pc1 and pc3 variants while we were able to detect large amounts of NP protein expressed from all the strains including D-del pc1 and pc3 variants. This indicates that the truncated NS1 viruses display major differences in their ability to express their mutant NS1 proteins, with high levels of expression for D-del pc2 and pc5 viruses, followed by delNS1 parent and D-del pc4 viruses (low levels) and finally by D-del pc1 and pc3 viruses (undetectable by Western blot).

The replication efficiency of the biologically purified NS variants was evaluated in Vero and CEF cells. Virus titers were measured by real-time RT-PCR at 24, 48, and 72 h post-infection. The titers of all

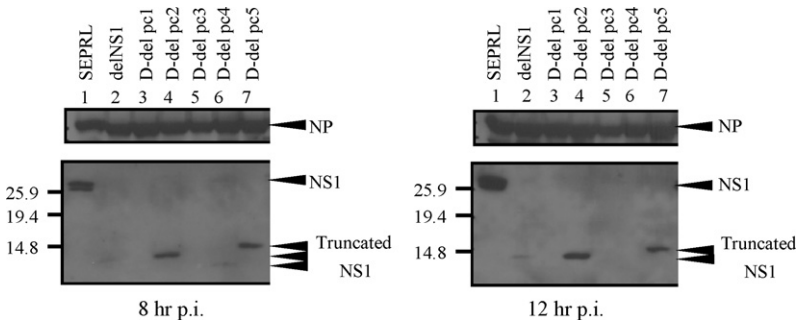

Fig. 3. Western blot analysis of the NS1 protein. Vero cells were infected with wild-type TK/OR/71 and derivative viruses for the indicated time points at an MOI of 2. Cell extracts were probed with an antibody against the NS1 or NP protein as described in Section 2.

variants and original delNS1 viruses were comparable to that of the wild type SEPRL virus at all time points analyzed. All variants tested reached their highest titers (10^6–10^7 EID_{50}/ml) at 48 h p.i. in Vero cells. All variants also replicated equally well and were comparable with the SEPRL and delNS1 virus in CEF cells reaching peak titers (approximately 10^7 EID_{50}/ml) by 24 h p.i. (data not shown).

3.4. *In vivo pathogenicity, replication, and transmission studies of selected NS variants*

We tested four biologically purified D-del NS variants (D-del pc1–pc4) in birds. The variants tested replicated poorly in 2-week-old SPF birds and only small amounts of virus were detected mainly at 2 days p.i. from a few infected birds (Table 1). All variants did not transmit the virus to contact control cage mates (determined by virus isolation and antibody response) and induced no clinical signs or histopathological lesions. We were not able to detect any viral antigen from any of the tissues examined including the trachea by immunohistochemical staining. It is clear contrast with the wild-type SEPRL virus which express full-length NS protein and showed efficient virus replication, virulence, and transmission to contact control birds in different ages of chickens [18]. Two variants, D-del pc3 and D-del pc4, were of particular interest because they replicated poorly in infected birds, but induced relatively high antibody titers at 2-week p.i. As shown in Table 1, only 1 out of 10 D-del pc3 variant infected birds shed low amounts of virus at 2 days p.i. From D-del pc4 infected birds, low amounts of virus in 3 birds at 2 days p.i. and 1 bird at 4 days p.i., respectively, was detected from tracheal swabs.

3.5. *Protective efficacy of potential live vaccines after challenge with heterologous strain of the same HA subtype*

Since low pathogenic avian influenza viruses seldom produces clinical signs in experimental conditions, protection efficacy was measured by the amount of virus shedding at 3 and 5 days post-challenge. In this protection study with a high dose heterologous virus challenge (10^6 EID_{50}/0.2 ml), live virus vaccination with D-del pc3 and D-del pc4 was highly effective by significantly reducing the amount of challenge virus shedding (Table 2). Although D-del pc1 and D-del pc2 vaccinated birds showed a certain level of protection, most birds still shed relatively large amount of the virus. The protection rate was correlated with the level of HI antibody titer measured at 2-week post-vaccination and 10 days post-challenge (Tables 1 and 2). All challenge-control birds shed high amounts of virus both at 3 and 5 days post-challenge (Table 2).

4. Discussion

The NS1 protein of influenza A virus is dispensable in certain conditions, as a recombinant virus lacking the entire NS1 coding region has been generated and shown to grow in IFN-deficient Vero cells [30]. However, the same virus could not replicate efficiently in IFN competent cells. In our study, we were able to rescue infectious viruses by reverse genetics that contained different D-del NS genes, except D-del 5 and 16 genes, as long as they retained the NEP splicing site. We could not rescue the reassortants that had one of nine D-del NS genes which lost the splicing site for NEP protein translation and part of the NEP coding sequence (Fig. 2b). NEP (or NS2) protein is an essential structural protein for virus replication and our results partly support the model of NS gene evolution proposed by Winter et al. [31]. In this model, the NS1 protein originally occupied the entire vRNA, but a second shorter mRNA encoding NEP was produced resulting from mutations which generated a splice site in the NS1 mRNA. At the same time, the NEP protein became more critical for the function of the virus than the carboxyl-terminal part of the NS1 protein, which was then progressively lost.

In our extensive trial, we were able to biologically clone five NS1 mutant virus variants (D-del pc1–pc5) by plaque purification in CEF cells, and confirmed that the NS genes of those variants were genet-

Table 1
Pathogenicity and transmission of D-del NS variants in 2-week-old SPF chickens

Group	Infected birds			Uninoculated cage mates		
	Virus isolation		HI titer (2 week PI[a])	Virus Isolation		HI titer (2 week PI)
	2 DPI[a]	4 DPI		2 DPI	2 DPI	
D-del pc1	7/10[b] (2.0 + 0.4)[c]	2/10 (2.3 + 1.2)	30 + 19	0/4	0/4	0
D-del pc2	1/10 (2.4)	0/10	16 + 0[d]	0/4	0/4	0
D-del pc3	1/10 (2.9)	0/10	50 + 41	0/4	0/4	0
D-del pc4	3/10 (2.6 + 0.6)	1/10 (2.0)	202 + 85	0/4	0/4	0

[a] Days post-infection.
[b] Number of birds positive/number tested.
[c] Virus titer is expressed as $\log_{10}$ median egg infective doses per milliliter.
[d] Only one bird showed measurable antibody titer.

Table 2
Protection of D-del NS variants infected birds after challenge with 10^6 EID_{50} of heterologous H7N2 virus

Group	Virus isolation		Average HI titer (10 DPC)
	3 DPC[a]	5 DPC	
D-del pc1	3/6[b] (3.2 + 0.5)[c]	5/6 (2.6 + 0.3)	133
D-del pc2	6/6 (3.8 + 1.4)	6/6 (3.2 + 0.9)	106
D-del pc3	2/6 (3.2 + 0.7)	0/6	2901
D-del pc4	1/6 (3.2 + 0.0)	1/6 (1.8)	1562
Unvaccinated Control	6/6 (4.7 + 0.7)	6/6 (3.3 + 0.5)	166

[a] Days post-challenge.
[b] Number of birds positive/number tested.
[c] Virus titer is expressed as $\log_{10}$ median egg infective doses per milliliter.

ically stable. It is likely that those NS genes had selective advantages in CEF cells compared to other NS genes in terms of replication and plaque forming efficiency. However, the selected variants express similar or more truncated NS1 protein compared to the NS1 protein of delNS1. In addition, none of the variants that retained the partial 3′ end effector domain were selected (Fig. 2b). Furthermore, the D-del pc1, which among the five variants has the largest deletion in its NS gene and thus expresses together with the D-del pc3 variant, the smaller NS1 proteins of the five variants, was the most predominant variant observed in our cloning and plaque purification study (data not shown). Thus, the size of the NS1 protein and selection efficiency did not correlate. It is possible that the dimerization function of the effector domain is sequence-specific and that the newly introduced sequences at the 3′ end of each NS1 protein due to deletion or frameshift influences the stability of the RNA binding domain and thus the function of the NS1 protein. The five biologically purified variants encode NS1 proteins of 86 (pc3), 90 (pc1), 93 (pc4), 125 (pc2) and 132 (pc5) amino acids, respectively. Although we were able to confirm the NS1 expression of some of those D-del variants by Western blot analysis, NS1 expressions from D-del pc1 and pc3 were not detected. In our growth curve study in Vero and CEF cells, all variants replicated comparably well and thus the replication efficiency does not appear to be a factor for NS1 detection from infected cells. It should be noted that the polyclonal NS1 antibody used in the Western blots was raised against a recombinant truncated NS1 protein of 73 amino acids, which are present in all six recombinant NS1 truncated viruses analyzed in these studies. Thus, it is also possible that the deletion in the 3′ carboxyl terminal region may have affected the three-dimensional structure of the 5′ RNA binding domain recognized by the antibody we used. The reason for the different levels of NS1 expression among the different NS1 truncated viruses remains unclear, but similar observations with other NS1 truncated viruses were previously reported [13].

In a study with NS1 truncated, mouse-adapted human influenza viruses, the length of the NS1 protein correlated with the level of the attenuation of these viruses: the shorter the NS1 protein, the less virulent is the virus [14]. However, in studies with NS1 truncated equine and swine influenza viruses, the degree of attenuation did not strictly correlate with the length of the NS1 protein [12,13]. In our study with avian influenza virus, we also found that degree of attenuation did not correlate with the length of NS1 protein. It is possible that different levels of attenuation of NS variants in different species may result from a different role of NS1 protein in different species. However, all these studies have been performed with a discrete number of NS1 variants from different viral strains. In addition, as shown by the X-ray crystal structure [32], the three-dimensional structure of the NS1 effector domain involves multiple interactions between amino acids that are far apart on the linear map of the NS1 protein effector domain. Thus, more investigations are required to determine the influence of the length in conjunction with the three-dimensional structure of the NS1 protein on its function and stability.

The NS1 protein of influenza A virus has several advantages as a target to attenuate influenza virus in developing a live-attenuated vaccine. However, there is also a concern that a live-attenuated vaccine strain may mutate and revert to the virulent wild-type. Truncation of the NS1 protein renders irreversible attenuation of influenza A virus and has been shown to be a valuable tool for safe influenza vaccine development [8,33,34]. Our *in vivo* study shows that all the selected variants (D-del pc1–pc4) are highly attenuated in chickens and despite the compensatory adaptations, none of the variants regain the original virulence of wild-type SEPRL virus that express intact NS1 protein [18]. Among the variants, D-del pc3 and pc4 viruses showed the most promising characteristics as a vaccine candidate strain. These variants replicated poorly in infected birds without transmitting the virus to contact control cage mates and produced no clinical signs or histopathological lesions. At the same time, the variants induced relatively high antibody titer at 2-week p.i. (Table 1). In a protection study with a high dose heterologous virus challenge, the live-attenuated variants vaccination was highly effective by reducing the amount of challenge virus shedding (Table 2). Therefore, these NS deletion variants can be excellent master strains for live-attenuated vaccine development. Additional introductions of mutations, such as changes that can convert the virus to a temperature-sensitive phenotype [35,36], or introducing immunostimulatory genes [37,38] in the deleted region of the NS gene may provide additional safety features.

In summary, we demonstrated that diverse NS genes of different sizes could be generated directly from the parental TK/OR/71-delNS1 strain. In addition, our *in vitro* and *in vivo* studies with selected isolates show that our naturally selected NS1 deletion variants might be useful in the development of a live influenza virus vaccines in their current state or with further modifications by reverse genetics. Furthermore, deletion in the NS1 protein can be potentially useful as a negative marker for a differentiating infected from vaccinated animals (DIVA) approach.

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